A COMPARATIVE STUDY OF INVERTASE PREPARATIONS FROM DIFFERENT SOURCES

DISSERTATION

Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in the Faculty of Pure Science,
at Columbia University

BY

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ACKNOWLEDGMENT

To Professor John M. Nelson the author wishes to express her appreciation for suggesting the problem and for his interest and advice throughout the work.

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INTRODUCTION

The question of the identity of invertase from different sources has been an open one for some time. Purification studies[1] have not reached the stage where one can, after following a given procedure, claim to have the enzyme free from impurities nor can one by analytic means attempt a differentiation between various preparations. In such a case it becomes necessary to make comparisons by indirect means. In attacking such a problem one must select reliable criteria and preferably ones which are independent of each other. Previous efforts have been made, notably those of Kuhn,[2] who claimed that yeast invertase was different from Taka invertase (from Aspergillus oryzae) because the former hydrolyzed raffinose as well as sucrose while the latter preparation was effective only with sucrose. He suggested that the yeast invertase hydrolyzed sucrose by combining with the fructose part of the molecule, which would permit the hydrolysis of raffinose, but that the Taka invertase combined with the glucose part, thereby preventing the hydrolysis of raffinose in which the glucose is modified by the presence of the galactose group. Kuhn[3] also claimed that these two preparations differed even in their action on sucrose in that inversion by the enzyme from yeast was retarded by β glucose and by fructose but not by α glucose, while inversion by the enzyme from the mold was retarded by α glucose but not by β glucose nor by fructose. In addition he[2] claimed that invertases from different races of brewery and distillery yeast varied in their values of the affinity constant, defined by Michaelis and Menten,[4] of the enzyme for sucrose. Although these studies have furthered the idea that invertase from various sources may be different, only a few sources were investigated. Before undertaking the present investigation it was necessary to select definite and, if possible, simple experimental methods whereby the different enzyme preparations might be compared. Three such criteria have been chosen and they will be discussed in the order in which they are employed in the experimental section.

The first method involves the study of the characteristic reaction velocity curve of the hydrolysis of sucrose by invertase. The hydrolysis of sucrose when catalyzed by hydrogen ions appears to be a pseudo-monomolecular reaction but, when the hydrolysis is catalyzed by brewery yeast invertase, the values of k in the monomolecular equation are observed to increase[5] as the reaction progresses. Various attempts have been made to elaborate theoretical equations for sucrose hydrolysis but all have failed to describe the reaction exactly. Because of this fact, Nelson and Hitchcock,[6] using a ten per cent sucrose solution, plotted the per cent hydrolyzed against the elapsed time of the reaction and derived an empirical equation of the curve. This equation,

$$n = \frac{1}{t}\left(\log \frac{100}{100 - p} + 0.002642p - 0.0_5 8860p^2 - 0.0_6 1034p^3\right)$$

where t represents the time in minutes for p, the per cent hydrolyzed, was found to give constant values of n at different values of t and p for four of the six enzyme preparations studied. These four preparations were considered normal while the other two which gave slightly decreasing values of n during the course of a hydrolysis were called abnormal. Since that time many invertase preparations from brewery and bakery yeast have been studied in this regard and only very rare cases of abnormal enzymes, those which give decreasing values of n, have been found. Miss Carmichael[7] tried to make a normal preparation behave abnormally by subjecting it to changes of physical and chemical environment. Sixty to seventy per cent inactivation at 60° C. and pH = 6 proved to have no effect on the values of n, other than making them smaller due to lessening of enzymic activity, while inactivation by nitrous acid caused the values of n during a hydrolysis to decrease so slightly as to be of doubtful significance. Furthermore, as from time to time new steps have been added to the purification procedure, the preparations have been tested and found to remain normal.[8] In fact, it has proved practically impossible to change this kinetic characteristic by treatment of the extracted enzyme. Recently Fassnacht [9] investigated the effect of certain changes in the composition of the culture media of the growing yeast on the reaction velocity curve of the extracted invertase. He used a bakery yeast throughout his work and found that this property of the enzyme remained unchanged when the yeast was grown in a simple malt solution or in one with a low salt content and in the presence of either sucrose or glucose. When the yeast was grown in a malt medium of higher salt content, hydrolysis with the extracted invertase showed slightly decreasing values of n. However, work in progress at the present time in this laboratory indicates that, if the yeast is grown repeatedly in this medium, it apparently becomes acclimated and after awhile the invertase again becomes normal in giving constant values of n. It thus appears that the reaction velocity curve for sucrose hydrolysis, as described by the equation of Nelson and Hitchcock, is a very constant characteristic of invertase and that this property is unresponsive to nearly all of the attempts to change it. Hence, this equation would seem to be a reliable criterion for comparing invertase from different sources, the only ones previously investigated being brewery and bakery yeast and honey. Nelson and Cohn[10] have found that honey invertase gives values of n which increase greatly to a maximum and then decrease to below the first value.

The second standard for comparison is the retardation of sucrose hydrolysis by α methyl glucoside which has been found in this laboratory, in concentration of one per cent, to retard the action of normal brewery[7] and bakery[9] yeast invertase 73-78 per cent. A correlation has been developed between the normality of the invertase, as measured by the equation of Nelson and Hitchcock, and the value of the α methyl glucoside retardation. Normal preparations have been found to be retarded 73-78 per cent,[7, 9] but the preparations of Miss Carmichael [7] and of Fassnacht[9] which gave slightly decreasing values of n during the course of hydrolysis were retarded only 60-70 per cent. It has been shown that honey invertase does not follow this correlation exactly since Papadakis[11] has found that it is retarded only 20 per cent and Nelson and Cohn[10] have found that it gives very unusual values of n which were described in the preceding paragraph. Papadakis[11] has also found that grape invertase is not retarded at all, while Taka invertase is retarded only six per cent by α methyl glucoside in the hydrolysis of sucrose. The retarding effect of this glucoside on the hydrolytic action seems to indicate characteristic differences between invertase preparations from various sources and the broad zone of retardation (73-78 per cent) noted with yeast invertase, although possibly due to experimental error, may have been caused by the use of somewhat different strains of yeast, since none of the work was done according to any pure culture technic. The value of the α methyl glucoside retardation then is a second possible criterion for the comparison of different preparations of invertase.

The third comparison is based upon the relation between the pH of the hydrolyzing solution and the velocity of hydrolysis. Michaelis and co-workers[12, 13] have measured the velocity of the enzymic hydrolysis of sucrose at different hydrogen ion concentrations. These velocities, calculated as fractions of the maximum observed velocity, were plotted against the corresponding pH values. The curve obtained resembled the dissociation-residue curve of an ampholyte and exhibited in the more alkaline region, a point of inflection at half the maximum velocity. Considering only the alkaline portion of the curve, it was interpreted as being the dissociation-residue curve of the invertase-sucrose complex ionizing as a weak acid. If this interpretation is valid, then the hydrogen ion concentration corresponding to half the maximum velocity equals the dissociation constant of the complex as an acid. This constant has been reported by Michaelis and Rothstein[13] as $10^{-6.5}$ at 24.5° C. and by Nelson and Bloomfield[14] as $10^{-7.0}$ at 25° C. Certain possible causes of this difference, other than the source of the enzyme, are discussed in the second part of the experimental section. Whether or not this theoretical interpretation is justified, the experimental determination of the pH activity relationships of different preparations may serve as an additional method of comparing invertase from different sources. Further work on the effect of pH on the reaction indicates that there is an optimal zone of hydrogen ion concentration where maximum activity is observed and that this optimum is constant for invertase from a given source but may be different when invertase from a different source is tested. Table I cites some of the optimal pH zones for invertase from several sources.

TABLE I.

Optimal pH zones for invertase from several sources.

Source of invertase	pH
Aspergillus oryzae[3, 15, 16, 17, 18]	4.6–6.0
Honey[10]	5.5–6.3
Pneumococcus[19]	7
Hemolytic streptococcus[20]	7
Penicillium glaucum[21]	4–6
14 species of leaves[22]	all vary
Yeast[13]	4.5–5.0

The value given for yeast invertase is for brewery yeast but no attempt has previously been made to study invertase from pure cultures of yeast. The value of the optimal pH region and the acid dissociation constant of the enzyme-substrate complex may then be taken as a third criterion for comparison.

It has been the purpose of the present investigation to further the study of invertase as a cell product. A survey of the properties of invertase from different sources was undertaken and comparison made between the results obtained with closely allied races of yeast and with more widely different sources. Table I has shown that the optimum pH for invertase action depends upon the source of the enzyme. The optimal pH zones of four different pure cultures of yeast have been determined in order to ascertain whether the value quoted for yeast invertase is the same for invertase from all yeasts or whether it is an average of values which vary with the strain of yeast used. PH-activity curves were plotted, for the same reason, with these preparations. Further modifications of the growth conditions were studied in relation to their effect on the invertase produced by the cells under varying conditions of aeration, pH, and buffer content of the medium. In the case of a yeast which gave an abnormal invertase various methods of extraction of the enzyme from the cells and of purification were used in order to determine whether the preparation used possessed the true characteristics of invertase from that strain of yeast or whether its properties had become modified on freeing it from the cells. The significance of the study will rest in tracing the characteristics of the extracted invertase back to the source from whence it was taken by determining if the properties of this enzyme are constant when it is obtained from a single source and if variations in these properties are specific for the different source materials.

EXPERIMENTAL

Part I

Comparison of invertase from different sources by means of the equation of Nelson and Hitchcock and of the α methyl glucoside retardation.

Two criteria by which the different enzyme preparations have been compared are the constancy of the term, n, in the equation of Nelson and Hitchcock [6] and the percentage retardation of the enzyme activity in ten per cent sucrose solution by one per cent α methyl glucoside. As sources of invertase four* races of yeast, one mold, one monilia, and raisins have been used. The yeasts have all been grown on the following standard liquid medium at room temperature.

1	liter unhopped malt (sp. gr. = 1.025 — 1.030)
12	grams primary ammonium phosphate
1	gram potassium chloride
1	gram calcium sulphate
1	gram magnesium sulphate
150	grams sucrose
3	liters distilled water

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* Before the work on S. pastorianus could be completely checked, the strain was lost, and the fresh cultures obtained from several stock culture companies failed to contain any invertase.

This medium was found by Fassnacht [9] to give a normal invertase with Fleischmann's yeast, that is, an invertase which gave constant values of n and approximately 78 per cent retardation with α methyl glucoside. The initial pH of this medium after sterilization was found to be 4.5 and consequently suitable for the growth of yeast. For details of the preparation of each invertase solution, see *History of Enzyme Preparations.*

Table IIa presents the data for the equation of Nelson and Hitchcock and Table IIb, the data for the retardations, the hydrolyses for which were carried out within the optimal pH zones for each preparation as determined in Part II.

TABLE II.

a—Comparison of invertase preparations by means of the equation of Nelson and Hitchrock.

Hydrolyses using invertase C and C' from S. cerevisiae.

Δt in minutes	R°	ΔR°	Per cent hydrolyzed	$n \times 10^5$
0	15.54			
90	11.63	3.91	19.38	156.3
150	9.24	6.30	31.15	155.1
210	6.95	8.59	42.48	156.4
270	4.96	10.58	52.33	155.9
330	3.18	12.36	61.13	156.1
420	0.95	14.59	72.16	157.4
450	0.34	15.20	75.17	157.7
				mean = 156.8
				average deviation = 0.5%
0	15.57			
60	10.98	4.59	22.70	276.6
120	6.92	8.65	42.78	276.0
150	5.14	10.43	51.57	275.6
180	3.57	12.00	59.34	274.9
210	2.16	13.41	66.32	275.5
240	0.95	14.62	72.32	276.4
270	—0.09	15.66	77.46	274.2
				mean = 275.6
				average deviation = 0.2%

TABLE II-a (continued)

Hydrolyses using invertase E and E′ from S. ellipsoideus.

Δt in minutes	R°	ΔR°	Per cent hydrolyzed	$n \times 10^5$
0	15.55			
60	7.24	8.31	41.10	527.2
81	4.91	10.64	52.62	520.0
100	3.04	12.51	61.86	523.7
120	1.38	14.17	70.07	524.9
140	0.04	15.51	76.70	526.1

mean = 524.4

average deviatoin = 0.4%

0	15.55			
60	9.89	5.66	27.99	345.5
96	6.96	8.59	42.48	342.1
120	5.19	10.36	51.22	341.6
150	3.30	12.25	60.57	339.2
180	1.60	13.95	69.00	341.6
210	0.26	15.29	75.60	341.4
240	—0.83	16.38	81.00	342.6

mean = 342.0

average deviation = 0.3%

Hydrolyses using invertase V and V′ from S. validus.

Δt in minutes	R°	ΔR°	Per cent hydrolyzed	$n \times 10^5$
0	15.57			
180	9.34	6.23	30.81	127.7
240	7.54	8.03	39.71	126.8
300	5.92	9.65	47.72	125.4
360	4.44	11.13	55.02	124.5
420	3.14	12.43	61.46	123.6

mean = 125.6

average deviation = 1.0%

0	15.58			
60	9.40	6.18	30.56	379.8
91	6.72	8.86	43.81	374.0
120	4.58	11.00	54.40	368.2
150	2.70	12.88	63.71	363.9
180	1.19	14.39	71.15	358.7

mean = 368.9

average deviation = 1.7%

Hydrolyses using invertase P from S. pastorianus.

Δt in minutes	R°	ΔR°	Per cent hydrolyzed	$n \times 10^5$
0	15.54			
120	7.73	7.81	38.63	245.5
150	6.07	9.47	46.83	245.3
180	4.58	10.96	54.21	244.4
210	3.26	12.28	60.73	243.1
270	1.00	14.54	71.92	243.4
300	0.11	15.43	76.30	243.1

mean = 244.1
average deviation = 0.37%

Δt in minutes	R°	ΔR°	Per cent hydrolyzed	$n \times 10^5$
0	15.55			
60	8.94	6.61	32.69	408.7
90	6.14	9.41	46.53	405.8
110	4.50	11.05	54.64	403.9
130	3.03	12.52	61.92	403.4
150	1.75	13.80	68.25	403.9
170	0.66	14.89	73.65	402.6

mean = 404.5
average deviation = 0.4%

Hydrolyses using invertase M and M' from Monilia.

Δt in minutes	R°	ΔR°	Per cent hydrolyzed	$n \times 10^5$
0	15.54			
20	12.82	2.72	13.45	481.9
90	4.87	10.67	53.14	476.6
120	2.35	13.19	65.23	470.8
150	0.42	15.12	74.79	468.8
180	−1.01	16.55	81.84	467.2

mean = 473.1
average deviation = 1.02%

Δt in minutes	R°	ΔR°	Per cent hydrolyzed	$n \times 10^5$
0	15.54			
20	14.21	1.33	6.58	232.8
59	11.74	3.80	18.80	230.8
118	8.29	7.25	35.86	230.0
140	7.17	8.37	41.39	227.7
207	4.10	11.44	56.58	224.5
265	1.96	13.58	67.16	222.5
315	0.48	15.06	74.48	221.9

mean = 227.2
average deviation = 1.5%

TABLE II-a (continued)

Hydrolyses using invertase R from raisins.

Δt in minutes	R°	ΔR°	Per cent hydrolyzed	$n \times 10^6$
0	15.51			
240	14.19	1.32	6.53	192.5
600	12.23	3.28	16.22	194.9
1322	8.55	6.96	34.42	196.3
1980	5.32	10.19	50.40	203.0
2790	2.04	13.47	66.62	208.8
3335	0.19	15.32	75.77	215.9

mean = 201.9

average deviation = 3.7%

Δt in minutes	R°	ΔR°	Per cent hydrolyzed	$n \times 10^6$
0	15.49			
180	13.80	1.69	8.36	329.4
360	12.14	3.35	16.57	332.2
610	9.97	5.52	27.30	331.0
1270	4.69	10.80	53.41	340.0
1440	3.48	12.01	59.40	344.1
1860	0.72	14.77	73.05	362.8

mean = 341.6

average deviation = 2.8%

Hydrolyses using invertase T and T′ from Aspergillus oryzae.

Δt in minutes	R°	ΔR°	Per cent hydrolyzed	$n \times 10^6$
0	15.44			
265	10.07	5.37	26.56	739.9
425	7.33	8.11	40.11	724.0
540	5.62	9.82	48.57	711.7
720	3.38	12.06	59.64	691.9
916	1.52	13.92	68.83	668.5
1080	0.41	15.03	74.32	643.9

mean = 696.6

average deviation = 4.1%

Δt in minutes	R°	ΔR°	Per cent hydrolyzed	$n \times 10^5$
0	15.52			
60	12.72	2.80	13.85	165.6
120	10.32	5.20	25.72	157.9
180	8.52	7.00	34.62	145.1
240	6.77	8.75	43.27	139.8
300	5.28	10.24	50.62	134.7
360	4.06	11.46	56.69	129.4
500	1.42	14.10	69.73	125.0

mean = 142.5

average deviation = 8.3%

TABLE II (continued)

b—Comparison of invertase by means of α methyl glucoside retardations.

Hydrolyses using invertase C and C′ from S. cerevisiae.

Unretarded				Retarded			
Δt in minutes	R°	ΔR°	Velocity	Δt in minutes	R°	ΔR°	Velocity
0	15.55			0	19.31		
33	15.19	0.36	0.0109	63	19.21	0.10	0.0016
63	14.86	.69	.0110	93	19.14	.17	.0018
93	14.54	1.01	.0109	124	19.06	.25	.0020
123	14.23	1.32	.0107	153	19.03	.28	.0018
153	13.89	1.66	.0108	183	18.99	.32	.0017
			mean = 0.0109				mean = 0.0018
					Retardation = 83%		

Unretarded				Retarded			
Δt in minutes	R°	ΔR°	Velocity	Δt in minutes	R°	ΔR°	Velocity
0	15.55			0	19.33		
33	15.11	0.44	0.0136	33	19.24	0.09	0.0027
63	14.68	.87	.0138	63	19.15	.18	.0029
93	14.28	1.27	.0137	93	19.07	.26	.0028
123	13.89	1.66	.0135	123	19.02	.31	.0025
153	13.52	2.03	.0133	153	18.95	.38	.0025
183	13.12	2.43	.0133	183	18.88	.45	.0025
			mean = 0.0135				mean = 0.0026
					Retardation = 81%		

Hydrolysis using invertase E and E′ from S. ellipsoideus.

Unretarded				Retarded			
Δt in minutes	R°	ΔR°	Velocity	Δt in minutes	R°	ΔR°	Velocity
0	15.49			0	19.23		
28	15.06	0.43	0.0154	27	19.09	0.14	0.0052
57	14.69	.80	.0140	57	18.93	.30	.0053
87	14.27	1.22	.0140	87	18.83	.40	.0046
117	13.82	1.67	.0143	117	18.66	.57	.0049
147	13.37	2.12	.0144	147	18.47	.76	.0052
177	12.96	2.53	.0143	177	18.37	.86	.0049
			mean = 0.0144				mean = 0.0050
					Retardation = 65%		

TABLE II-b (continued)

Hydrolysis using invertase E and E′ from S. ellipsoideus.

Unretarded				Retarded			
Δt in minutes	R°	ΔR°	Velocity	Δt in minutes	R°	ΔR°	Velocity
0	15.52			0	19.23		
30	15.09	0.43	0.0143	30	19.07	0.16	0.0053
60	14.65	.87	.0145	64	18.94	.29	.0045
90	14.23	1.29	.0143	90	18.76	.47	.0052
120	13.80	1.72	.0143	120	18.56	.67	.0056
150	13.38	2.14	.0143	150	18.49	.74	.0049
180	12.96	2.56	.0142	180	18.34	.89	.0049
			mean = 0.0143				mean = 0.0051

Retardation = 64%

Hydrolyses using invertase V and V^{iv} from S. validus.

Unretarded				Retarded			
Δt in minutes	R°	ΔR°	Velocity	Δt in minutes	R°	ΔR°	Velocity
0	15.51			0	19.27		
33	15.25	0.26	0.0079	30	19.24	0.03	0.0010
63	14.98	.53	.0084	60	19.15	.12	.0020
93	14.70	.81	.0087	90	19.12	.15	.0017
123	14.40	1.11	.0090	120	19.07	.20	.0017
153	14.10	1.41	.0092	150	19.05	.22	.0015
183	13.80	1.71	.0093	180	19.01	.26	.0014
			mean = 0.0088				mean = 0.0016

Retardation = 82%

0	15.46			0	19.39		
4	15.07	0.39	0.098	40	18.57	0.82	0.0205
8	14.62	.84	.105	60	18.15	1.24	.0207
12	14.22	1.24	.103	80	17.73	1.66	.0208
16	13.82	1.64	.102	100	17.31	2.08	.0208
20	13.42	2.04	.102				mean = 0.0207
			mean = 0.102				

Retardation = 80%

TABLE II-b (continued)

Hydrolysis using invertase P from S. pastorianus.

Unretarded				Retarded			
Δt in minutes	R°	ΔR°	Velocity	Δt in minutes	R°	ΔR°	Velocity
0	15.57			0	19.31		
33	15.20	0.37	0.0112	63	19.16	0.15	0.0024
93	14.53	1.04	.0112	93	19.04	.27	.0029
123	14.16	1.41	.0115	123	18.96	.35	.0028
153	13.81	1.76	.0115	153	18.92	.39	.0025
183	13.49	2.08	.0114	183	18.89	.42	.0023
		mean	= 0.0114			mean	= 0.0026
					Retardation = 77%		

Hydrolyses using invertase M and M′ from Monilia.

Unretarded				Retarded			
Δt in minutes	R°	ΔR°	Velocity	Δt in minutes	R°	ΔR°	Velocity
0	15.54			0	19.25		
30	15.325	0.215	0.0072	30	19.21	0.04	0.0013
62	15.13	.41	.0066	60	19.16	.09	.0015
90	14.88	.66	.0073	90	19.15	.10	.0011
120	14.69	.85	.0071	120	19.13	.12	.0010
150	14.52	1.02	.0068	150	19.05	.20	.0013
180	14.31	1.23	.0068	180	18.995	.255	.0014
		mean	= 0.0070			mean	= 0.0013
					Retardation = 81%		
0	15.53			0	19.255		
42	15.06	0.47	0.0112	60	19.16	0.095	0.0016
66	14.81	.72	.0109	90	19.11	.145	.0016
93	14.52	1.01	.0109	120	18.99	.265	.0022
123	14.20	1.33	.0108	150	18.92	.335	.0022
153	13.81	1.72	.0112	180	18.865	.390	.0022
183	13.47	2.06	.0113				
						mean	= 0.0020
		mean	= 0.0110				
					Retardation = 82%		

A summary of the results of these hydrolyses is given in Table III to facilitate comparisons.

TABLE III.

Comparison of Invertase from Different Sources.

Source of invertase	Values of n	Per cent retardation by α methyl glucoside
S. cerevisiae	constant	82
S. ellipsoideus	constant	65
S. pastorianus	constant	77
S. validus	decreasing	81
Monilia	decreasing	81
Aspergillus oryzae	decreasing	*
Raisins	increasing	*
Honey	†	*

* See Papadakis[11]
Aspergillus oryzae—retarded approximately 6%
Grapes—not retarded
Honey—retarded approximately 20%.

† See Nelson and Cohn[10]
Values of n increase to a maximum and then fall off.

The hydrolysis data show that the kinetics of invertase action varies with the source of the enzyme material, since the values of n may be constant, increasing or decreasing and since the retardation due to α methyl glucoside is not always the same. However, with one race of yeast grown under identical conditions, reproducible values of each factor are observed.

On closer examination the results further reveal that the differences between invertase from various yeasts are only slight. The values of n are either constant or else exhibit only very slight increases or decreases—not more than two per cent if calculated as the average deviation from the mean value. Likewise, all of the yeast invertases which have been studied are strongly retarded by α methyl glucoside, the lowest retardation being sixty-five per cent. But if we go to a different type of source material, such as molds, more highly organized plants, or animals,* we find great differences from the yeast preparations. Invertase preparations from Aspergillus oryzae and from honey are only slightly retarded by α methyl glucoside and that from grapes, not at all. The enzyme from the mold gives greatly decreasing values of n, that from raisins strongly increasing, whereas that from the animal source gives a great rise followed by a great decrease in its value.

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* The invertase present in honey is secreted by the bee.

In former investigations[7, 9] constancy of the value of n has been correlated with an α methyl glucoside retardation of 73-78 per cent, while preparations giving decreasing values of n have given lower retardations of 60-65 per cent. These results have been obtained by treating normal invertase preparations or yeasts which give normal invertase so as to render them abnormal. Thus, the values represent work on invertase from the same source, usually Ruppert's[7] or Fleischmann's[9] yeast. In the present work, however, it is seen that this correlation no longer holds when we compare enzyme preparations from different sources. In Table III we find S. cerevisiae and S. pastorianus behaving in the traditional fashion, giving constant values of n and high retardations. But, contrary to this, S. ellipsoideus gives constant values of n and a low retardation. These particular results do not indicate what would happen to the retardation if the character of n of any one of these preparations were changed but they do show that there is no general correlation between the constancy of the term n and the value of the retardation by α methyl glucoside. In other words, if we take an invertase preparation of unknown origin or one which has not previously been studied, we cannot foretell the value of the retardation from the character of n nor the character of n from the value of the retardation.

The conclusions drawn from these experimental results are that each of the different source materials studied produces an invertase with certain definite kinetic properties, that these properties vary according to the source of the enzyme, and that invertases from specimens widely separated biologically differ to a much greater extent than do those from more closely allied organisms.

EXPERIMENTAL

Part II.

Comparison of pH-activity curves of invertase from different yeasts.

Earlier work has shown that the optimum pH for the activity of invertase varies, depending on the source of the enzyme. Among the source materials studied were yeast, molds, honey, pathogenic bacteria, animal intestine, and leaves. In this respect yeasts have not been studied in pure culture but only as brewery and bakery yeast. Even so, Michaelis and Rothstein[13] have reported a dissociation constant of $10^{-6.5}$ at 24.5° C. for their preparation while Nelson and Bloomfield [14] have reported the value of $10^{-7.0}$ at 25° C. for theirs. There are several factors which may have been responsible for the difference in the values. In the first place, the strains of yeast probably were not identical. Secondly, unpublished work [23] in this laboratory indicates that the buffer used affects the slope of the pH-activity curve and Michaelis and Rothstein used a series of acetate and phosphate buffers to cover their pH range while Nelson and Bloomfield used citrate buffers for theirs. Finally, recent work [23] has shown that the concentration of sucrose probably does affect the slope of this curve, contrary to the work of Michaelis and Rothstein. The value of the dissociation constant given by these authors is determined from a curve drawn through points representing hydrolyses in various sucrose concentrations, while the constant given by Nelson and Bloomfield is for 10 per cent sucrose solutions. To avoid these possible sources of error, in the following experiments 0.01 M citrate buffers and 10 per cent sucrose solutions were used throughout and only the strain of yeast was varied.

The determination of the optimum pH for activity of the different enzyme preparations was further necessitated by the fact that the α methyl glucoside retardation depends upon the pH at which the velocities are determined.[24]

Velocities of hydrolysis at different pH values were determined and the pH-activity curves plotted for the different yeast invertase preparations. In Figures I to IV the actual velocities of hydrolysis in degrees change in rotation per minute are plotted against the pH of the hydrolyzing solutions, while the curves in Figure V represent the relation between pH and enzymic activity when the maximum activity observed is considered as unity.

From these graphs the optimum pH of S. cerevisiae is found to be 4.5 to 5.0, that of S. pastorianus 3.9 to 4.3, that of S. ellipsoideus 4.2 to 4.7, and that of S. validus 4.5 to 5.0.

When the velocities are calculated as fractions of the maximum velocity and these relative velocities are plotted against pH, we have a curve similar to a dissociation residue curve of a weak acid and the determination of the hydrogen ion concentration corresponding to one-half of the maximum velocity will give the acid dissociation constant of the enzyme-substrate complex, according to the Michaelis theory.[12, 13] From the curves in Figure V, these dissociation constants are found to be $10^{-7.25}$ for S. cerevisiae, $10^{-7.4}$ for S. pastorianus, $10^{-6.8}$ for S. ellipsoideus, and $10^{-7.2}$ for S. validus.

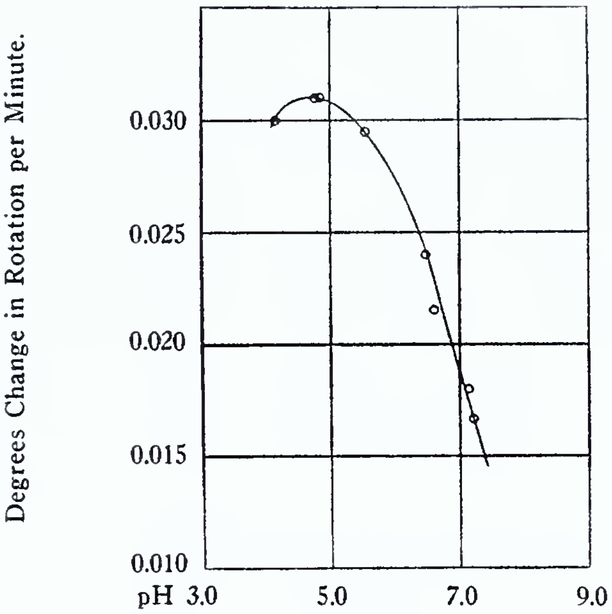

FIGURE I.
pH-activity curve of S. cerevisiae invertase.

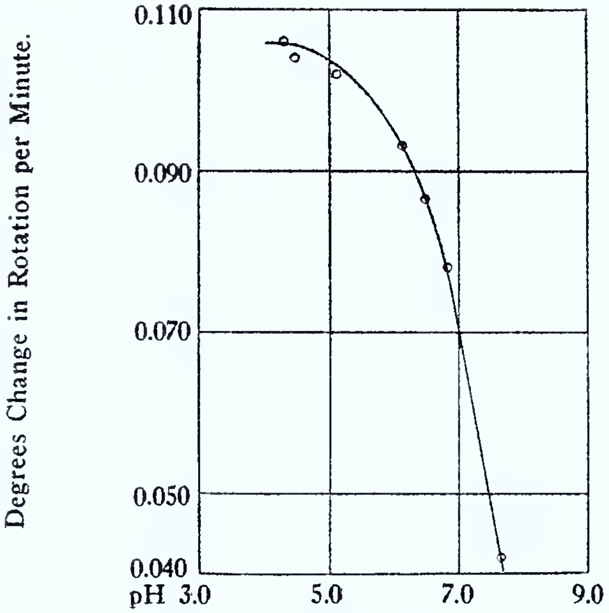

FIGURE II.

pH-activity curve of S. pastorianus invertase.

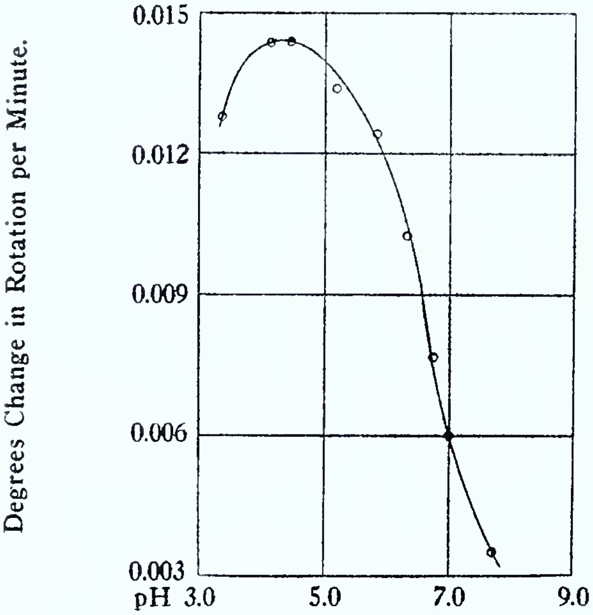

FIGURE III.

pH-activity curve of S. ellipsoideus invertase.

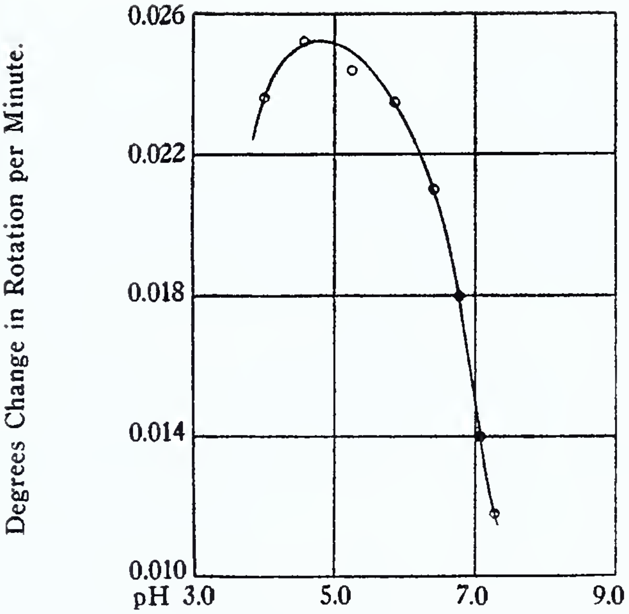

FIGURE IV.
pH-activity curve of S. validus invertase.

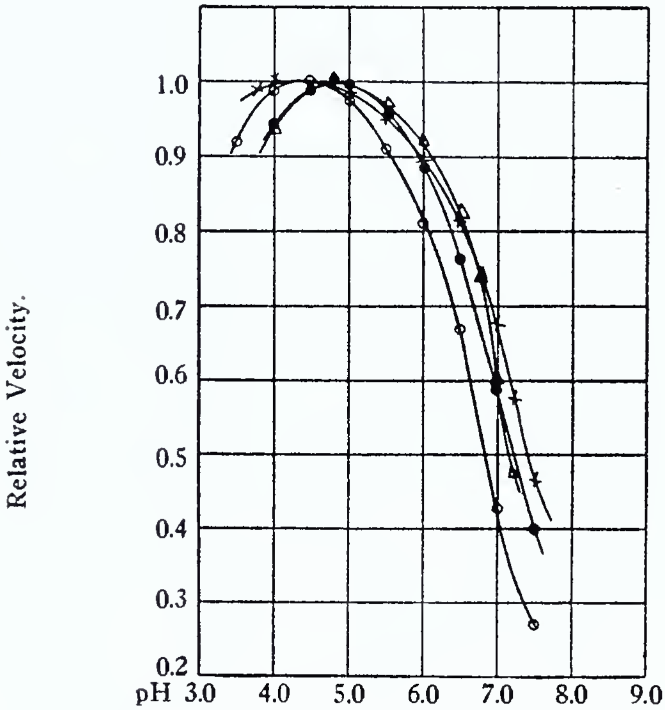

FIGURE V.
Relative pH-activity curves.

Key: O = S. ellipsoideus invertase ✕ = S. pastorianus invertase
△ = S. validus invertase • = S. cerevisiae invertase

The optimal pH values for yeast invertase appear to coincide rather closely as do the dissociation constants. However, there are slight differences. It should be noted that the yeasts studied here are all ones which may be encountered in the same habitat, S. cerevisiae being used in the manufacture of beer, while the other three cause disease, turbidity, or a poor tase in beer.[25] In other words, they are not widely different strains and consequently one would expect the differences among their products to be but slight.

EXPERIMENTAL

Part III.

A study of the effect of the growth conditions and of the method of obtaining the enzyme on the invertase produced by certain strains of yeast.

Since certain differences in kinetic behavior were found among the enzyme preparations from different yeasts, the decision was made to investigate some of the conditions of growth of the yeast and of extraction of the enzyme from the cells in relation to their effect on the invertase produced. The growth conditions studied include the aeration of the medium during growth, the initial pH of the growth medium, and the use of a more strongly buffered growth medium. The methods of extraction include the use of toluene and of chloroform as cell poisons causing autolysis and the mechanical method of extracting the enzyme by grinding the yeast with sand followed by extraction with water, thereby eliminating the autolysis procedure.

A—The effect of aeration of the medium during the growth of the yeast on the kinetics of the invertase produced.

Since S. validus invertase, as shown in Part I, gave decreasing values of n in the equation of Nelson and Hitchcock, it was chosen for these experiments. Transplants from the same culture were made into two flasks of standard malt media at room temperature. The one flask, containing culture V^{v}, was plugged with cotton and shaken occasionally, while through the other, containing culture V^{vi}, was drawn continuously a current of sterilized air. The two cultures were grown for the same length of time, the filtered yeast autolyzed with toluene, and the enzyme precipitated in 50 per cent ethyl alcohol and extracted with water. The hydrolysis data in Table IV show decreasing values of n in both cases, indicating that the aeration has produced no effect on the character of the velocity curve of sucrose hydrolysis by the extracted enzyme. The fact that invertase from aerated and non-aerated yeast are retarded in like degree by α-methyl glucoside further indicates that the aeration of the yeast produces no change in the behavior of the enzyme.

TABLE IV.

a—Comparison of invertase preparations by means of the equation of Nelson and Hitchcock.

Hydrolyses with invertase V^v from non-aerated S. validus.

Δt in minutes	R°	ΔR°	Per cent hydrolyzed	$n \times 10^5$
0	15.58			
60	11.11	4.47	22.11	269.2
121	7.13	8.45	41.79	266.5
150	5.50	10.08	49.85	264.5
180	3.99	11.59	57.32	262.6
211	2.63	12.95	64.05	260.7
240	1.54	14.04	69.44	258.7

mean = 263.7

average deviation = 1.15%

0	15.55			
70	11.25	4.30	21.27	221.7
141	7.40	8.15	40.31	219.5
175	5.79	9.76	48.27	218.0
210	4.30	11.25	55.64	216.5
240	3.16	12.39	61.28	215.4
270	2.15	13.40	66.27	214.1

mean = 217.5

average deviation = 1.01%

Hydrolyses with invertase V^{vi} from aerated S. validus.

Δt in minutes	R°	ΔR°	Per cent hydrolyzed	$n \times 10^5$
0	15.55			
50	9.57	5.98	29.57	439.9
70	7.48	8.07	39.91	437.0
90	5.60	9.95	49.21	433.9
110	3.94	11.61	57.42	430.6
130	2.50	13.05	64.54	427.7
150	1.30	14.25	70.47	423.8

mean = 430.5

average deviation = 1.12%

TABLE IV-a (continued)

Hydrolyses with invertase V^{vi} from aerated S. validus.

Δt in minutes	R°	ΔR°	Per cent hydrolyzed	$n \times 10^5$
0	15.61			
30	11.88	3.73	18.45	445.6
50	9.61	6.00	29.62	440.7
70	7.54	8.07	39.91	437.0
95	5.23	10.38	51.34	432.7
126	2.83	12.78	63.20	428.3
				mean = 436.9

average deviation = 1.2%

TABLE IV (continued)

b—Comparisom of invertase preparations by means of α methyl glucoside retardations.

Hydrolysis with invertase V^{v} from non-aerated S. validus.

Unretarded				Retarded			
Δt in minutes	R°	ΔR°	Velocity	Δt in minutes	R°	ΔR°	Velocity
0	15.55			0	19.23		
7	14.85	0.70	0.100	44	18.33	0.90	0.0205
11	14.40	1.15	.105	64	17.91	1.32	.0206
15	14.00	1.55	.103	84	17.49	1.74	.0207
19	13.60	1.95	.103	104	17.07	2.16	.0208
23	13.20	2.35	.102				
			mean = 0.103				mean = 0.0206

Retardation = 80%

Hydrolysis with invertase V^{vi} from aerated S. validus.

Unretarded				Retarded			
Δt in minutes	R°	ΔR°	Velocity	Δt in minutes	R°	ΔR°	Velocity
0	15.53			0	19.27		
5	15.30	0.23	0.0460	30	18.99	0.28	0.0093
10	15.07	.46	.0460	55	18.77	.50	.0091
15	14.84	.69	.0460	90	18.46	.81	.0090
22	14.50	1.03	.0468	130	18.09	1.18	.0091
29	14.18	1.35	.0466	190	17.54	1.73	.0091
44	13.50	2.03	.0461				
			mean = 0.0462				mean = 0.0091

Retardation = 80%

B—The effect of varying the initial pH of the standard media on the kinetics of the invertase produced.

It is known that the buffer content of the growth medium is of considerable importance in the production of toxin by the diphtheria bacillus.[26] If the media is only slightly buffered so that at the beginning of growth the pH can fall and then later rise to 8.0 or higher, a very potent toxin is obtained from the culture when the pH is on this last increase. On the other hand, if the media is so highly buffered that the typical pH-time curve is not followed, a very weak toxin if any is produced. It was thought possible that the character of the invertase from a strain of yeast might likewise be affected by the buffer content of the media since invertase is, also, a product of a unicellular organism. The diphtheria toxin is, of course, an exo-toxin while invertase has been considered an endo-enzyme.

Before testing out the effect of the buffer content of the media it seemed advisable to ascertain whether or not varying the initial pH of the standard media would produce any change in the properties of the invertase obtained. S. cerevisiae was chosen for this study because the brewery and bakery yeast invertase preparations which have been most thoroughly investigated probably consist largely of various strains of this yeast and, also, because this treatment would be more apt to produce a change towards abnormality than in the opposite direction.

The pH of the several batches of media were roughly adjusted before sterilizing and the exact pH determined electromet-

TABLE V.

Data on Titration Curve of 10 cc. of Normal Media.

Cc. N/2 HCl	pH
0.00	6.04
.24	5.86
.37	5.66
.55	4.88
.84	3.19
.95	2.85
1.05	2.63
1.18	2.40
1.28	2.39
1.79	2.10

rically after sterilizing. The most alkaline medium was titrated with N/2 HCl to determine the value of the media as a buffer. The titration data are given in Table V and the graph of the titration curve in Figure VI.

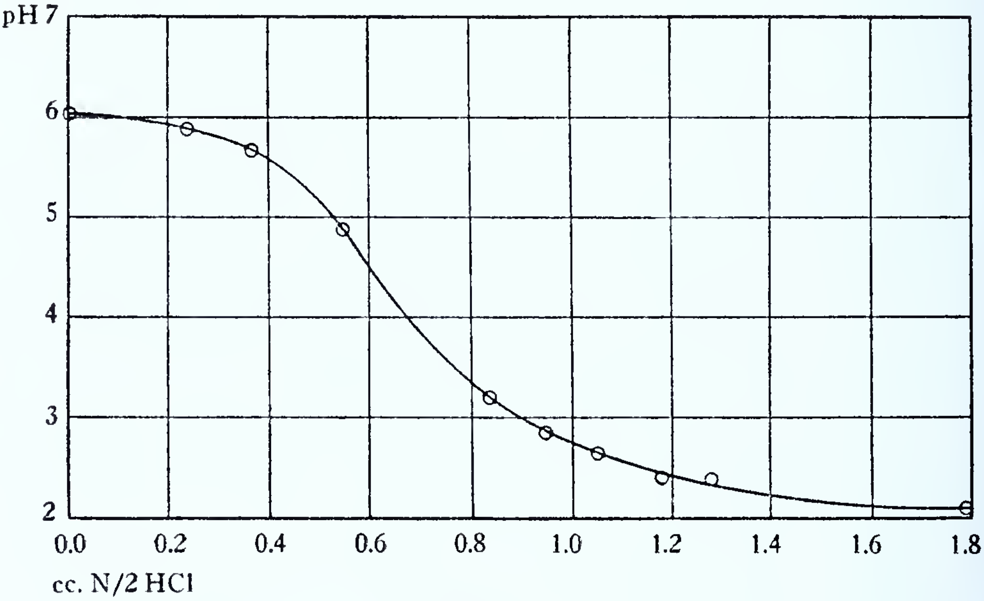

FIGURE VI.

Titration curve of 10 cc. normal media against N/2 HCl.

After the media had been sterilized and its initial pH determined, each batch was inoculated with S. cerevisiae and grown for nine days at room temperature. The yeast was then filtered off and the final pH of the cultures determined. This is not the true final pH of the strain since the yeast was still alive and fer-

TABLE VI.

Optimal pH zone for growth of S. cerevisiae.

Time elapsed	Initial pH of media						
in days	2.53	3.12	3.79	4.54	4.80	5.15	6.04
2	±	+	+++	++	++	+	—
3	—	+	+++	++	+++	++	+
4	—	+	++	+++	+++	+++	+
9	—	+	++	+++	+++	+++	++

+++ = greatest growth; ++ = medium growth; + = slight growth; — = no growth; ± = doubtful.

menting at the time when the cells were filtered off for autolysis. Table VI indicates the relative amounts of growth in the different flasks estimated by the amount of fermentation and the cloudiness of the media when shaken.

In Table VII are given the initial and final pH values of each culture and likewise, the percentage change in hydrogen ion concentration.

TABLE VII.

Change in pH during growth of S. cerevisiae.

Initial pH	Final pH	Per cent Change in $[H+]$
2.53	2.23	69
3.12	2.79	76
3.79	2.87	211
4.54	3.00	354
4.80	3.14	382
5.15	3.53	373
6.04	4.60	331

In the flask at initial pH = 2.53 no apparent growth took place but it was not determined whether the change in pH was due to fermentation by the cells of the inoculum or due to a natural change in the pH on standing in Pyrex glass, such as has been observed by Grace and Highberger[27] and others.[28]

The invertase was prepared from the yeast in the usual manner and hydrolyses were performed at the optimum pH to determine the values of n and the retardations.

TABLE VIII.

a—Comparison of invertase preparations by means of the equation of Nelson and Hitchcock.

Hydrolysis with invertase $C_{pH6.04}$ from S. cerevisiae grown at pH = 6.04

Δt in minutes	R°	ΔR°	Per cent hydrolyzed	$n \times 10^5$
0	15.57			
60	8.97	6.60	32.64	406.0
90	6.20	9.37	46.33	403.9
110	4.50	11.07	54.73	404.8
130	3.00	12.57	62.16	405.5
160	1.11	14.46	71.52	407.1
190	—0.40	15.97	79.00	410.6

mean = 406.3
average deviation = 0.4%

TABLE VIII-a (continued)

Hydrolysis with invertase $C^{pH4.54}$ from S. cerevisiae grown at pH = 4.54

Δt in minutes	R°	ΔR°	Per cent hydrolyzed	$n \times 10^5$
0	15.65			
60	8.91	6.74	33.34	417.5
90	6.03	9.62	47.58	416.7
120	3.58	12.07	59.69	415.6
150	1.54	14.11	69.78	417.1
180	—0.11	15.76	77.96	422.2

mean = 417.8

average deviation = 0.4%

Hydrolysis with invertase $C^{pH3.12}$ from S. cerevisiae grown at pH = 3.12

Δt in minutes	R°	ΔR°	Per cent hydrolyzed	$n \times 10^5$
0	15.55			
120	8.16	7.39	36.54	231.0
160	6.11	9.44	46.69	229.2
200	4.27	11.28	55.76	228.0
241	2.61	12.94	64.00	227.9
280	1.265	14.285	70.63	227.8
342	—0.45	16.00	79.11	228.7

mean = 228.8

average deviation = 0.4%

b—Comparison of invertase preparations by means of the α methyl glucoside retardations.

Hydrolysis with invertase $C^{pH6.04}$ from S. cerevisiae grown at pH = 6.04

Unretarded				Retarded			
Δt in minutes	R°	ΔR°	Velocity	Δt in minutes	R°	ΔR°	Velocity
0	15.44			0	19.25		
25	15.05	0.39	0.0156	25	19.19	0.06	0.0024
55	14.59	.85	.0155	55	19.12	.13	.0024
75	14.25	1.19	.0159	75	19.05	.20	.0027
95	13.90	1.54	.0162	95	18.99	.26	.0027
115	13.59	1.85	.0161	115	18.93	.32	.0028
135	13.26	2.18	.0161	135	18.90	.35	.0026

mean = 0.0159 mean = 0.0026

Retardation = 84%

TABLE VIII-b (continued)

Hydrolysis with invertase $C_{pH4.54}$ from S. cerevisiae grown at pH = 4.54

Unretarded				Retarded			
Δt in minutes	R°	ΔR°	Velocity	Δt in minutes	R°	ΔR°	Velocity
0	15.52			0	19.23		
7	14.83	0.69	0.099	42	18.60	0.63	0.0150
11	14.44	1.08	.098	64	18.25	.98	.0153
15	14.06	1.46	.097	82	17.94	1.29	.0157
19	13.69	1.83	.096	103	17.62	1.61	.0156
23	13.31	2.21	.096	125	17.25	1.98	.0158
			mean = 0.0972				mean = 0.01548
							Retardation = 84%

Hydrolysis with invertase $C_{pH3.12}$ from S. cerevisiae grown at pH = 3.12

Unretarded				Retarded			
Δt in minutes	R°	ΔR°	Velocity	Δt in minutes	R°	ΔR°	Velocity
0	15.48			0	19.24		
31	15.07	0.41	0.0132	25	19.18	0.06	0.0024
55	14.75	.73	.0133	45	19.10	.14	.0031
75	14.49	.99	.0132	65	19.08	.16	.0025
95	14.25	1.23	.0129	85	19.04	.20	.0024
115	14.03	1.45	.0126	102	18.99	.25	.0024
135	13.72	1.76	.0130	140	18.94	.30	.0021
			mean = 0.0130				mean = 0.0025
							Retardation = 81%

The results of these experiments presented in Table VIII indicate that the quality of the invertase extracted from S. cerevisiae is independent of the initial pH of the media, although the initial pH does affect the yield of yeast and also the amount of fermentation as represented by the drop in pH during growth. It should, however, be noticed in this connection that in the region of pH = 3.8-5.0 the media is not a very good buffer so that the same amount of fermentation would cause a greater pH drop in this region than in the more alkaline or more acid parts of the curve. It was further observed that there was a large amount of invertase in the yeast grown at pH = 6.04 and pH = 4.54, but only a small amount in that at pH = 3.12. A quantitative study of the amount of invertase produced by yeast in solutions of different acidity has been made by Euler and Svanberg[29] with similar results but the quality of such preparations has not been previously investigated.

C—The effect of partially buffered growth media on the characteristics of yeast invertase.

Since it was shown in Part III-B that the initial pH of the medium does not affect invertase from S. cerevisiae, this yeast was chosen to carry on the work with buffered media. Four media have been used in this connection:

1—normal media containing 0.25 per cent of calcium carbonate,

2—normal media containing N/10 ammonium carbonate,

3—normal media containing M/10 primary potassium phosphate,

4—normal media containing M/20 primary potassium phosphate and M/20 secondary sodium phosphate.

For details of these preparations, reference should be made to *History of Enzyme Preparations.* Table IX contains the hydrolysis data for these preparations.

TABLE IX.

a—Comparison of invertase preparations by means of the equation of Nelson and Hitchcock.

Hydrolysis with invertase C_{CaCO_3} from S. cerevisiae

Δt in minutes	R°	ΔR°	Per cent hydrolyzed	n × 10^5
0	15.66			
50	11.27	4.39	21.71	316.8
80	8.86	6.80	33.63	316.2
110	6.69	8.97	44.36	313.9
140	4.73	10.93	54.06	313.1
175	2.75	13.91	63.85	312.9
230	0.28	15.38	76.06	315.3

mean = 314.7
average deviation = 0.44%

TABLE IX-a (continued)

Hydrolysis with invertase $C_{(NH_4)_2CO_3}$ from S. cerevisiae

Δt in minutes	R°	ΔR°	Per cent hydrolyzed	n × 10^5
0	15.59			
45	7.90	7.69	38.03	643.9
61	5.59	10.00	49.46	644.0
75	3.82	11.77	58.21	643.1
85	2.68	12.91	63.84	644.0
95	1.66	13.93	68.90	645.7
105	0.76	14.83	73.35	647.2
117	—0.17	15.76	77.91	648.7

mean = 645.2
average deviation = 0.29%

Hydrolysis with invertase $C_{KH_2PO_4}$ from S. cerevisiae

Δt in minutes	R°	ΔR°	Per cent hydrolyzed	n × 10^5
0	15.55			
120	9.66	5.89	29.13	180.4
180	7.06	8.49	41.99	180.0
240	4.74	10.81	53.46	180.1
300	2.75	12.80	63.30	180.3
360	1.08	14.47	71.56	181.1

mean = 180.4
average deviation = 0.16%

Hydrolysis with invertase $C_{KH_2PO_4-Na_2HPO_4}$ from S. cerevisiae

Δt in minutes	R°	ΔR°	Per cent hydrolyzed	n × 10^5
0	15.58			
25	10.12	5.46	27.00	798.0
35	8.15	7.43	36.75	797.1
45	6.32	9.26	45.80	796.4
55	4.64	10.94	54.10	797.8
70	2.74	13.11	64.84	799.7
85	0.71	14.87	73.54	803.1

mean = 798.7
average deviation = 0.22%

TABLE IX (continued)

b—Comparison of invertase preparations by means of α methyl glucoside retardations.

Hydrolysis with invertase C_{CaCO_3} from S. cerevisiae

Unretarded				Retarded			
Δt in minutes	R°	ΔR°	Velocity	Δt in minutes	R°	ΔR°	Velocity
0	15.54			0	19.24		
4	15.19	0.35	0.0875	25	18.88	0.36	0.0144
8	14.84	.70	.0875	45	18.59	.65	.0144
12	14.48	1.06	.0883	65	18.29	.95	.0146
16	14.14	1.40	.0875	85	17.99	1.25	.0147
20	13.77	1.77	.0885	105	17.69	1.55	.0148
24	13.43	2.11	.0879				
							mean = 0.0146
			mean = 0.0879				

Retardation = 83%

Hydrolysis with invertase $C_{(NH_4)_2CO_3}$ from S. cerevisiae

Unretarded				Retarded			
Δt in minutes	R°	ΔR°	Velocity	Δt in minutes	R°	ΔR°	Velocity
0	15.55			0	19.26		
5	15.00	0.55	0.110	20	18.87	0.39	0.0195
10	14.44	1.11	.111	40	18.46	.80	.0200
15	13.90	1.65	.110	60	18.07	1.19	.0198
20	13.35	2.20	.110	80	17.67	1.59	.0199
25	12.82	2.73	.109	100	17.29	1.97	.0197
			mean = 0.110				mean = 0.0198

Retardation = 82%

Hydrolysis with invertase $C_{KH_2PO_4}$ from S. cerevisiae

Unretarded				Retarded			
Δt in minutes	R°	ΔR°	Velocity	Δt in minutes	R°	ΔR°	Velocity
0	15.43			0	19.25		
7	15.05	0.38	0.054	25	19.03	0.22	0.0088
14	14.70	.73	.052	50	18.81	.44	.0088
21	14.35	1.08	.051	85	18.50	.75	.0088
28	14.02	1.41	.050	115	18.21	1.04	.0090
35	13.66	1.77	.051	145	18.00	1.25	.0086
			mean = 0.052				mean = 0.0088

Retardation = 83%

TABLE IX-b (continued)

Hydrolysis with invertase $CKH_2PO_4-Na_2HPO_4$ from S. cerevisiae

Unretarded				Retarded			
Δt in minutes	R°	ΔR°	Velocity	Δt in minutes	R°	ΔR°	Velocity
0	15.55			0	19.25		
5	14.64	0.91	0.1820	25	18.49	0.76	0.0304
10	13.73	1.82	.1820	35	18.185	1.065	.0304
15	12.81	2.74	.1827	50	17.71	1.54	.0308
20	11.92	3.63	.1815	65	17.25	2.00	.0308
				80	16.75	2.50	.0312
		mean =	0.1820			mean =	0.0307

Retardation = 83%

Table X summarizes these results.

TABLE X.

Comparison of the properties of invertase from S. cerevisiae grown in partially buffered media.

Type of media	pH drop during growth	Values of n	Per cent retardation by 1% α methyl glucoside
Normal	4.5–3.0	constant	82
1— “ —$CaCO_3$	5.9–5.1	constant	83
2— “ —$(NH_4)_2CO_3$	7.7–6.0	constant	82
3— “ —KH_2PO_4	4.5–4.0	constant	83
4— “ — “ + Na_2HPO_4	6.8–6.0	constant	83

From these results it is clear that a pure culture of S. cerevisiae is not affected by high salt concentrations as is the Fleischmann's yeast used by Fassnacht[9] for in media 2, 3 and 4 the salt concentration is much higher than that used by Fassnacht who obtained an invertase preparation which showed slightly decreasing values of n and a slightly lowered retardation with α methyl glucoside. Furthermore, it is apparent that invertase from S. cerevisiae remains normal even when the yeast is grown in a more highly buffered medium, whether the buffering is at the optimum pH or at a somewhat more alkaline value.

D—The effect of toluene autolysis and of alcohol precipitation on the kinetics of the invertase produced.

In Tables I and IV it is seen that, if the cells of S. validus be autolyzed by adding toluene to a water suspension of the yeast and the invertase precipitated from the crude autolysate in fifty per cent ethyl alcohol and extracted from this precipitate with water, decreasing values of n are obtained from the hydrolysis data. In order to eliminate the possibility of the precipitation by alcohol having any effect on the character of the invertase, hydrolyses were performed using the crude toluene autolysate without further purification as enzyme material. The data in Table XI indicate that the crude enzyme shows the same characteristics as the purified.

TABLE XI.

a—Hydrolysis, followed by means of the equation of Nelson and Hitchcock, using crude toluene autolysate V^{vii} of S. validus.

Δt in minutes	R°	ΔR°	Per cent hydrolyzed	n × 10^6
0	16.02			
210	11.28	4.74	23.44	817.7
354	8.38	7.64	37.78	812.5
450	6.66	9.36	46.29	806.7
570	4.78	11.24	55.59	796.8
690	3.13	12.89	63.75	791.7
860	1.19	14.83	73.34	790.0

mean = 802.6

average deviation = 1.21%

b—α methyl glucoside retardation using crude toluene autolysate V^{vii} of S. validus.

Unretarded				Retarded			
Δt in minutes	R°	ΔR°	Velocity	Δt in minutes	R°	ΔR°	Velocity
0	15.57			0	19.27		
20	15.05	0.52	0.0260	65	18.98	0.29	0.0045
35	14.68	.89	.0254	125	18.72	.55	.0044
50	14.32	1.25	.0250	185	18.44	.83	.0045
65	13.94	1.63	.0251	245	18.16	1.11	.0045
90	13.35	2.22	.0247	305	17.87	1.40	.0046
			mean = 0.0252				mean = 0.0045

Retardation = 82%

E—The effect of chloroform autolysis on the kinetics of the invertase produced.

To some of the S. validus cells grown for use in Part III-D chloroform was added and the yeast autolyzed for the same length of time as when the toluene was used. Hydrolyses were then effected with the crude chloroform autolysate. Decreasing values of n were also obtained in this case for which the data appears in Table XII:

TABLE XII.

a—Hydrolysis, followed by means of the equation of Nelson and Hitchcock, using crude chloroform autolysate V^{viii} of S. validus.

Δt in minutes	R°	ΔR°	Per cent hydrolyzed	$n \times 10^6$
0	15.98			
300	11.23	4.75	23.49	573.7
427	9.37	6.61	32.69	574.3
550	7.72	8.26	40.85	571.2
790	4.88	11.10	54.90	565.9
970	3.11	12.87	63.65	562.0
1200	1.24	14.74	72.90	560.4

mean = 567.9
average deviation = 0.9%

b—α methyl glucoside retardation using crude chloroform autolysate V^{viii} of S. validus.

Unretarded				Retarded			
Δt in minutes	R°	ΔR°	Velocity	Δt in minutes	R°	ΔR°	Velocity
0	15.56			0	19.27		
25	15.22	0.34	0.0136	60	19.10	0.17	0.0028
45	14.96	.60	.0133	182	18.81	.46	.0025
65	14.69	.87	.0134	300	18.50	.77	.0026
86	14.40	1.16	.0135	420	18.21	1.06	.0025
185	13.12	2.44	.0132	540	17.94	1.33	.0025
			mean = 0.0134				mean = 0.0026

Retardation = 81%

F—The effect of mechanical extraction on the kinetics of the invertase produced.

Since both of the autolysates in Parts III-D and E failed to give a normal invertase preparation, it was thought that by departing from the chemical methods of freeing the enzyme one might obtain a preparation which behaved normally with respect to its velocity curve. Therefore, the cells of S. validus were ground with sand and the invertase extracted immediately with water. However, hydrolyses performed with this extract likewise appeared abnormal as may be seen from the data in Table XIII.

TABLE XIII.

a—Hydrolysis, followed by means of the equation of Nelson and Hitchcock, using mechanically extracted invertase V^{ix} from S. validus.

Δt in minutes	R°	ΔR°	Per cent hydrolyzed	$n \times 10^5$
0	15.56			
45	10.29	5.27	26.06	427.0
60	8.71	6.85	33.88	424.2
80	6.78	8.78	43.42	421.1
100	5.05	10.51	51.98	417.4
125	3.19	12.37	61.18	412.6
160	1.06	14.50	71.71	408.8

mean = 418.5

average deviation = 1.3%

b—α methyl glucoside retardation using mechanically extracted invertase V^{ix} from S. validus.

Unretarded				Retarded			
Δt in minutes	R°	ΔR°	Velocity	Δt in minutes	R°	ΔR°	Velocity
0	15.55			0	19.26		
5	15.03	0.52	0.104	20	18.86	0.40	0.020
10	14.51	1.04	.104	40	18.38	.88	.022
15	13.96	1.59	.106	60	18.00	1.26	.021
20	13.45	2.10	.105	80	17.66	1.60	.020
25	12.93	2.62	.105	100	17.06	2.20	.022

mean = 0.105 mean = 0.021

Retardation = 80%

Summing up the results included in Part III, it is seen that the kinetic properties under consideration are constant characteristics of invertase from a given race of yeast. These properties have been shown to be stable with regard to variations in the growth conditions of the yeast and also in respect to the methods of extracting the enzyme from the organisms. The fact that these properties do remain constant under such a variety of conditions lends additional support to the claim that the differences noted in Parts I and II are significant and do characterize the invertases obtained from the different sources.

EXPERIMENTAL DETAILS

History of invertase preparations

The normal media used throughout the greater part of the work contained

1 liter unhopped malt (sp. gr. = 1.025 - 1.030)
12 grams primary ammonium phosphate
1 gram potassium chloride
1 gram calcium sulphate
1 gram magnesium sulphate
150 grams sucrose
3 liters distilled water.

In certain cases additional ingredients were included as indicated.

Four liters of media, in Pyrex flasks plugged with cotton, were sterilized by boiling on two consecutive days. Control flasks, left uninoculated, remained sterile. The medium was inoculated by means of a sterilized platinum loop from stock pure cultures kept growing on agar slants of the normal media. The flasks were kept at room temperature, about 22° C., and after sufficient yeast was grown, the medium was filtered through fine filter paper on a Buchner funnel. The yeast was, unless otherwise indicated, autolyzed by the addition of toluene to a water suspension of the yeast. The invertase was precipitated, except where noted, in 50 per cent ethyl alcohol and extracted from this precipitate with water. Further details of each preparation are given below.

C—Saccharomyces cerevisiae, a top yeast used in brewing. "Numerous races and species of yeasts are known under the name of S. cerevisiae. Their systematic position is slightly known.—A large number of industrial species (brewery and distillery) related to these species have been described. Some produce a top fermentation while others produce a bottom fermentation."[25] A single strain of this yeast was used.

C—Grown two days in normal media without aeration, autolyzed for five days.

C'—Grown three days in normal media without aeration, autolyzed for twenty-seven days.

$C^{pH=6.04}$—Grown nine days in normal media, the pH of which was adjusted to 6.04, without aeration, autolyzed for four days. During growth the pH fell from 6.04 to 4.60.

$C^{pH=4.54}$—Same treatment as $C^{pH=6.04}$ except that the initial pH was 4.54. During growth the pH fell to 3.00.

$C^{pH=3.12}$—Same treatment as $C^{pH=6.04}$ except that the initial pH was 3.12. During growth the pH fell to 2.79.

C^{CaCO_3}—Grown eight days in normal media, to which ten grams of $CaCO_3$ were added, wiithout aeration but with continual shaking to ensure uniform environment, autolyzed for six days. Precautions were taken to prevent contamination during shaking. During growth the pH dropped from 5.9 to 5.1.

$C^{(NH_4)_2CO_3}$—Grown for five days in normal media, containing N/10 $(NH_4)_2CO_3$, without aeration, autolyzed for five days. During growth the pH fell from 7.7 to 6.0.

$C^{KH_2PO_4}$—Grown for ten days in normal media, containing N/10 KH_2PO_4, without aeration, autolyzed for nine days, and the extract from the alcohol precipitation dialyzed one day to remove color. During growth the pH fell from 4.5 to 4.0.

$C^{KH_2PO_4-Na_2HPO_4}$—Grown for eight days in normal media containing M/20 KH_2PO_4 and M/20 Na_2HPO_4, without aeration, autolyzed for six days. During growth the pH fell from 6.8 to 6.0.

V—Saccharomyces validus, "a top yeast isolated by Hansen from a bottom fermentation in beer where it caused cloudiness."[25]

V—Grown for two days in normal media without aeration, autolyzed for seven days.

V^{iv}—Grown for five days in normal media without aeration, autolyzed for seven days.

V^{v}—Grown for six days in normal media without aeration, autolyzed for five days.

V^{vi}—Grown for six days in normal media with aeration, autolyzed for five days.

V^{vii}—Grown for six days in normal media without aeration, autolyzed for three days in toluene and water, autolysate not purified.

V^{viii}—The same culture as V^{vii}, autolyzed for three days in chloroform and water, autolysate not purified.

V^{ix}—Grown as V^{vii}, cells were ground in a mortar with sand and invertase extracted with water but not purified.

E—Saccharomyces ellipsoideus turbidans, ordinarily a bottom yeast but may be transformed into a top yeast, causes diseased beer.[25]

E—Grown in normal media without aeration, autolyzed with toluene and water, invertase precipitated in 50 per cent ethyl alcohol and extracted with water, growth and autolysis times unknown.

E′—Grown in normal media for six days without aeration, autolyzed for ten days.

P—Saccharomyces pastorianus, contributes bitter disagreeable taste and bad odor to beer—contributes to diseases of beer and impedes clarification. Occurs partly in scum and partly in sediment.[25]

P—Grown two days in normal media with aeration, autolyzed for five days.

M—Monilia, in liquid media vegetates at first in sediment and later produces flocs in liquid and a scum like a mold.[25]

M—Grown seven days on normal media without aeration, autolyzed seven days.

M′—Grown six days on normal media without aeration, autolyzed five days.

T—Aspergillus oryzae, preparations of "Takadiastase" supplied by Parke, Davis & Co.

T—Four ounces of "Takadiastase" extracted with 300 cc. of saturated $(NH_4)_2SO_4$ for eight days, dialyzed until free from

$SO_4^=$, concentrated to 1/5 volume by Giemsa[30] ultrafiltration method.

T′—Same procedure as T but with a later preparation of "Taka-diastase."

R—"Sunmaid Seedless Raisins."

R—Raisins ground in meat grinder, invertase extracted in saturated ammonium sulfate, dialyzed free from $SO_4^=$, concentrated by Giemsa method.[30]

Method of performing hydrolyses

In all cases 50 cc. of enzyme solution were pipetted into 200 cc. of 12½ per cent sucrose solution containing 0.01M citrate buffer at the desired pH and at 25° ± 0.01° C. At recorded time intervals 25 cc. samples of the hydrolyzing solution were removed and to each sample was added a drop of sodium hydroxide solution of such concentration as to raise the pH to 8-9. This caused the hydrolysis to cease and hastened mutarotation. After mutarotation, the samples were polarized at 25° C. in a two dm. tube in a Schmidt and Haensch polarimeter, using light from a mercury arc of wave-length 546.1 $\mu\mu$. In the case of the retarded hydrolyses, the 200 cc. of sucrose solution also contained 1.25 per cent α methyl glucoside. The total change in rotation of a ten per cent sucrose solution is 20.22°.

Determination of pH

Since most of the solutions to be tested were highly colored and contained a considerable amount of protein material, the pH values were determined by measuring the emf. with the quinhydrone electrode and the saturated calomel half-cell on a "Type K" potentiometer. Where the composition of the solutions permitted, the values were checked by using the hydrogen electrode.

SUMMARY

In the present investigation three kinetic properties have been used to characterize a number of invertase preparations. It has been found that these properties remain constant for invertase from a given source, although the organisms producing the enzyme have been grown under different conditions and although different methods of extracting the enzyme have been employed. On this account we are brought to the conclusion that the determination of these properties does actually describe invertase from a given source. On the other hand, it has been found that these properties vary with invertase from different sources. These two facts indicate that each species of organism which hydrolyzes sucrose produces an invertase with definite kinetic characteristics but that one invertase may differ in these relationships from invertase from other species of organisms. It must be noted that the properties studied relate only to the efficiency of the enzyme as a catalyst and do not actually prove differences in either composition or structure of the different invertase preparations. The investigation further discloses that organisms closely allied biologically produce invertases with only slightly different properties, while the more widely separated species produce invertases which differ to a much greater extent.

REFERENCES

[1] Oppenheimer, C. and Pincussen, L., Die Methodik der Fermente, 1929, p. 767.

[2] Kuhn, R., Z. physiol. Chem. **125** 28 (1923).

[3] Kuhn, R., Z. physiol. Chem. **129** 57 (1923).

[4] Michaelis, L. and Menten, M. L., Biochem. Z. **49** 333 (1913).

[5] Weidenhagen, R. and Dey, B. B., Z. Ver. deut. Zuckerind. **1928** 242.

[6] Nelson, J. M. and Hitchcock, D. I., J. Am. Chem. Soc. **43** 2632 (1921).

[7] Carmichael, H. M., Columbia University Dissertation, 1930.

[8] Unpublished work in this laboratory.

[9] Fassnacht, H. H., Columbia University Dissertation, 1930.

[10] Nelson, J. M. and Cohn, D. J., J. Biol. Chem. **61** 193 (1924).

[11] Papadakis, P. E., Columbia University Dissertation, 1929.

[12] Michaelis, L. and Davidsohn, H., Biochem. Z. **35** 387 (1911).

[13] Michaelis, L. and Rothstein, M., Biochem. Z. **110** 217 (1920).

[14] Bloomfield, G., Columbia University Dissertation, 1922.

[15] Anonymous, Jap. Med. Literature **5** 44 (1920).

[16] Leibowitz, J. and Mechlinski, P., Z. physiol. Chem. **154** 64 (1926).

[17] Weidenhagen, R., Z. Ver. deut. Zuckerind, **1928** 125.

[18] Hattori, Y., Biochem. Z. **150** 150 (1924).

[19] Avery, O. T. and Cullen, G. E., J. Exp. Med. **32** 547 (1920).

[20] Stevens, F. A. and West, R., J. Exp. Med. **35** 823 (1922).

[21] von Euler, H., Josephson, K., and Soderling, B., Z. physiol. Chem. **139** 1 (1924).

[22] Blagoveshenskii, A. V. and Sossiedov, N. I., Biochem. J. **19** 350 (1925).

[23] Nelson, J. M. and Palmer, A. H., unpublished work.

[24] Nelson, J. M. and Schubert, M., unpublished work.

[25] Guilliermond, A., The Yeasts, tr. and rev. by Tanner, F. W., N. Y., 1920.

[26] Bunker, J. W. M., J. Bact. **4** 379 (1919).

[27] Grace, L. G. and Highberger, F., J. Inf. Dis. **26** 457 (1920).

[28] Esty, J. R. and Cathcart, P. H., J. Inf. Dis. **29** 29 (1921).

[29] von Euler, H. and Svanberg, O., Z. physiol. Chem. **106** 201 (1919).

[30] Giemsa, G., Biochem. Z. **132** 488 (1922).

VITA

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